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(54) Title: COMPOUNDS WHICH INHIBIT HIV REPLICATION

(57) Abstract

    This invention relates to human immunodeficiency virus (HIV) protein fragments which have antiviral activity, and particularly relates to HIV peptides derived from the HIV transmembrane glycoprotein (gp41) which inhibit HIV-induced cell-cell fusion. This invention further relates to methods for the inhibition of enveloped viral infection, and to methods that modulate biochemical processes which involve coiled coil peptide interactions.

COMPOUNDS WHICH INHIBIT HIV REPLICATION

The present invention was made with Government
support under grants numbers R01-AI30411 and P30-
5   AI28662 from the National Institute of Allergy &
Infectious Diseases. The Government has certain
rights to this invention.

This application is a continuation-in-part of co-
10 pending application Serial No. 07/927,532 of C. Wild,
T. Matthews and D. Bolognesi, filed 7 August 1992, the
disclosure of which is to be incorporated by reference
herein in its entirety.

15   1. Introduction
This invention relates to human immunodeficiency
virus (HIV) protein fragments which have antiviral
activity, and particularly relates to HIV peptides
derived from the HIV transmembrane glycoprotein (gp41)
20 which inhibit HIV-induced cell-cell fusion. This
invention further relates to methods for the
inhibition of enveloped viral infection, and to
methods that modulate biochemical processes which
involve coiled coil peptide interactions.
25

                  2. Background of the Invention
Numerous HIV protein fragments, or peptides, have
been identified in an effort to develop an effective
HIV vaccine. See generally B. Spalding, Biotechnology
30 10, 24 (Jan. 1992). Examples of patent applications
which are directed to antigenic epitopes of the gp41
protein include J. Rosen et al., PCT Application WO
87/06005 and R. Duncan, EPO Application 0 371 817. To
date, the development of an anti-HIV vaccine has been
35 difficult.

N. Qureshi et al., Aids 1990 4, 553-558, describe
a segment of the HIV transmembrane protein (designated
"gp41") which inhibits T-cell activation in vitro.
This segment, designated "CS3", when conjugated to
5   human serum albumin and labeled with fluorescein,
binds specifically to CD4+ cell lines, and is said to
have antiviral activity. CS3 comprises amino acids
581 to 597 of the gp41 protein.
      B. Kemp et al., EPO Application 0 323 157,
10 describes a fragment comprised of amino acids 572 to
591 of the gp41 protein which is said to have
antiviral activity.

                3.   Summary of the Invention
15   A first aspect of the present invention is a
peptide selected from the group consisting of: (a) the
peptide DP-107, which has the formula, from amino
terminus to carboxy terminus, of:
            NNLLRAIEAQQHLLQLTVWGIKQLQARILAVERYLKDQ
20 (SEQ ID NO: 1); and (b) peptides of from 14 to 60
amino acids in length which form a heterodimer with
the peptide DP-107 (SEQ ID NO: 1) (hereinafter on
occasion referred to as "active compounds").
      A second aspect of the present invention is a
25 process for inhibiting HIV-induced cell fusion. The
process comprises contacting to an HIV-infected cell
an effective fusion-inhibiting amount of a peptide
selected from the group consisting of: (a) the peptide
DP-107, which has the formula, from amino terminus to
30 carboxy terminus, of:
            NNLLRAIEAQQHLLQLTVWGIKQLQARILAVERYLKDQ
(SEQ ID NO: 1); and (b) peptides of from about 14 to
60 amino acids in length which form a heterodimer with
the peptide DP-107 (SEQ ID NO: 1).
35

A third aspect of the present invention is a
process for testing compounds for the ability to
inhibit the ability of HIV to infect cells. The
process comprises (a) contacting a test compound to a
5   multimer of a peptide selected from the group
consisting of: (i) the peptide DP-107, which has the
formula, from amino terminus to carboxy terminus of:
        NNLLRAIEAQQHLLQLTVWGIKQLQARILAVERYLKDQ
(SEQ ID NO: 1) ; and (ii) peptides of from 14 to 60
10 amino acids in length which form a heterodimer with
the peptide DP-107 (SEQ ID NO: 1); and then (b)
detecting whether the test compound disrupts said
multimer, the ability of the test compound to disrupt
the multimer indicating the test compound is capable
15 of inhibiting HIV infection of cells.
        A further aspect of the invention is a method for
inhibiting enveloped viral infection comprising
contacting an uninfected cell with an effective amount
of a peptide capable of contributing to the formation
20 of a coiled coil peptide structure so that an
enveloped virus is inhibited from infecting the
uninfected cell.
        The foregoing and other objects and aspects of
the present invention are explained in detail in the
25 drawings herein and the specification set forth below.

        4.   Brief Description of the Drawings
        Figure 1 shows the sequences of various peptides
studied. In DP-107 (SEQ ID NO: 1), DP-121 (SEQ ID NO:
30 2), and DP-125 (SEQ ID NO: 3) the leucine or
isoleucine heptad repeat units are underlined. DP-
107, DP-121, and DP-125 are acetylated at the NH_2
terminus and amidated at the COOH terminus. DP-116
(SEQ ID NO:4) (identical to the CS3 peptide) is
35 amidated at the carboxy terminus and has a free amine

terminus. DP-31 (SEQ ID NO: 5) is neither acetylated
nor amidated. Amino acid residues are numbered
according to Human Retroviruses and AIDS (1991).

Figure 2 shows the CD spectra of 10 mM solutions
5   of DP-107(m), DP-121(+), and DP-116(1) at 0°C (A) and
37°C (B). Concentration dependence of the midpoint of
the temperature dependence (T_m) of the CD signal (C).
T_m corresponds to the maxima of the first derivative
of the CD melt curve. The CD spectra were obtained in
10 10mM sodium phosphate, 150mM sodium chloride buffer at
pH 7.0.

Figure 3 shows the test for peptide blockade of
AA5 cell infection by HIV- 1_{LAI} About 500 $TCID_{50}$ HIV-LAI
were added to 2×10^4 AA5 cells and test peptides (final
15 concentrations shown) in a final volume of 100 ml.
Cell cultures were maintained in 96-well microtiter
plates for 8 days by addition of fresh medium (but no
further addition of peptides) every other day. On the
eighth day post-infection, supernatant was tested for
20 reverse transcriptase activity as evidence of
successful infection.

Figure 4 shows the test for direct virocidal
effect of peptides and soluble CD4 (sT4) The HIV-1_{LAI}
virus stock was divided into two portions. Samples of
25 one portion (shown in figure as virus pelleted +) were
treated for 2 hr at 37°C in medium alone, with DP-107
at 40 mg/ml, or with sT4 at 10 mg/ml. Virus was then
pelleted through a 5% sucrose layer to separate virus
from non-associated inhibitor. The virus-containing
30 pellets were dispersed in media and serial dilutions
tested for infectivity on the AA5 cells. Serial four
fold dilutions of the other portion of virus (virus
pelleted -) were tested directly for infection of the
cells with each dilution of virus incubated in the
35 absence or presence of DP-107 at 40 mg/ml.

Figure 5 shows a comparison of peptides and
soluble CD4 to inhibit primary virus isolates. About
25 $TCID_{100}$ of two primary isolates and HIV-1_{LAI} were
added to PRA activated human PBMCs (about $1.5x10^6$/ml)
5   containing the indicated final concentrations of test
peptide or 5T4. Each treatment condition was tested
in duplicate and the cells were cultured in RPMI1640
containing 10% FCS 5% IL2. Four days post infection
an equal number of fresh PBMCs were added to each
10   well. This step allows for further expansion of virus
and increases the noise to background ratio in the
final RT assay. Supernatant was harvested 8 days
after infection and tested for the presence of reverse
transcriptase activity

15

          5.   Detailed Description of the Invention
          The term "HIV" as used herein refers to HIV-1,
and the numbering of amino acids in HIV proteins and
fragments thereof given herein is with respect to the
20   HIV1_{LAI} isolate. It is to be understood, however, that
while HIV viral infection and the effects of DP-107 on
such HIV infection are being used herein as a model
systems in which the potential anti-viral properties
of peptides capable of forming coiled coils are
25   described, such properties of coiled coil peptides may
represent generalized mechanisms by which a broad
spectrum of enveloped viral infections may be
inhibited. Enveloped viruses whose infectivity may be
inhibited using the coiled coil peptides of the
30   invention may include, but are not limited to, other
HIV strains such as HIV-2, as well as influenza
viruses, syncytial respiratory viruses, an herpes
viruses.
          The DP107 peptide sequence is based on a highly
35   conserved region in the transmembrane protein (TM)

- 6 -

which was predicted by Gallaher et al., AIDS Res. and
Human Retro. 5, 431 (1989), to form an extended
amphipathic α-helix with structural analogues in the
TM proteins of several fusogenic viruses such as
5 influenza and other retroviruses. The function of the
site is not known but may be related to
multimerization of the envelope glycoprotein. The
site has been shown to contain a "leucine zipper"
repeat. See E. Delwart et al., AIDS Res. and Human
10 Retro. 6, 703 (1990). The use of peptides such as DP-
107 capable of forming coiled coils, therefore, may
serve to interfere, block, or in some way modulate
many biochemical processes which involve such coiled
coil peptide formations. Such biochemical process may
15 include, but are not limited to transcription factors
(Abel and Maniatis, Nature 341:24) and physiological
processes involving membrane fusion (White, J.M.,
1992, Science 258:1917-1924).

    The biological activity of the peptide DP-107 was
20 unexpected and its mechanism is not readily apparent.
The results shown herein suggest that it does not act
directly on the cell-free virions. Qureshi et al.
(AIDS 4, 553 (1990)) have reported that an overlapping
peptide, CS3 (DP-116 herein), inhibited infection when
25 coupled to albumin and suggested that this occurred by
attachment to a second receptor on the cell surface
required for membrane fusion. These investigators
have tentatively identified a candidate for the
receptor as a 44 kD protein. Although such a
30 mechanism would be consistent with the DP-107 result
shown in Fig. 4 described hereinbelow, other
observations argue that these two peptides are quite
dissimilar and as such might function through
different mechanisms. Most importantly, the CS3
35 peptide was only active after conjugation to albumin

which contrasts with the striking anti-viral effect of
the free (non-conjugated) DP-107 peptide. Also, the
CS3 peptide showed no evidence for stable secondary
structure by CD even at low temperatures and high
5   concentrations. Our experiments indicate that
structure or capacity to assume stable secondary
structure is a requirement for biological activity.
For example, the DP-107 analogue containing a helix
breaking proline substitution (DP-121) and several
10   truncated versions of DP-107 (not shown) that either
disrupted or failed to show stable solution structure
did not exhibit anti-viral activity.

As noted above, a first aspect of the present
invention is a peptide selected from the group
15   consisting of:

(a)   the peptide DP-107, which has the formula,
from amino terminus to carboxy terminus, of:
NNLLRAIEAQQHLLQLTVWGIKQLQARILAVERYLKDQ (SEQ ID NO:1);
and

20   (b)   peptides of from 14 to 60 amino acids in
length which form a heterodimer with the peptide DP-
107 (SEQ ID NO:I).

In general, the peptide may be of any suitable
length, but is preferably from 14 to 60 amino acids in
25   length, and more preferably from 16 to 38 amino acids
in length. In addition, it will be appreciated that
minor variations can be made to the peptide. For
example, the peptide may be acetylated at the amino
terminus thereof and/or amidated at the carboxy
30   terminus thereof.

Peptides of the invention may be provided as
multimers, particularly as dimers and tetramers. When
provided in such form the multimer may be stabilized
by covalently joining the monomers to one another.
35   For example, a cysteine residue may be added to

either (or both) ends of the monomer and monomers of
the multimer covalently joined to one another by a
disulfide bond between cysteine residues. Reactions
are carried out in accordance with known techniques.

5 In this manner two monomers of a dimer may be
covalently joined to form a covalently stabilized
dimer, and if desired two such covalently stabilized
dimers conjugated to one another to form a tetramer.
In another example, all four members of a tetramer

10 could be covalently joined to one another through
disulfide linkages between terminally positioned
cysteine residues.

Other techniques for stabilizing the multimeric
forms of these peptides include crosslinking the

15 monomer components to one another through the
formation of intermolecular amide bonds. This process
involves the reaction of the amine moiety of a basic
amino acid residue i.e. lysine, with the carboxy
moiety of an acidic amino acid residue i.e. aspartic

20 or glutamic acid.

Several techniques can used to determine the
multimerization state of a given peptide or peptide
mixture (homodimer or heterodimer). The most
straightforward methods involve determining the

25 apparent molecular weight of the multimer complex and
from this determining the number of associated monomer
components (this can be accomplished by dividing this
apparent molecular weight by the molecular weight of
the monomer). Analytical ultracentrifugation is a

30 particularly suitable technique for this purpose. The
specifics of this method are known to those skilled in
the art. See., e.g., P. Graceffa et al., J. Biol.
Chem. 263, 14196-14202 (1988), and can be summarized
as follows. The material of interest is placed in a

35 sample cell and spun very rapidly in a model E

ultracentrifuge equipped with the appropriate
detection devices. Information collected during the
experiment combined with the amino acid composition of
the peptide allows for the determination of the
5   apparent MW of the multimer complex. Fast Protein
Liquid Chromatography (FPLC) can also be used for this
purpose. This technique is different from the above
in that, as a type of chromatography, it ultimately
requires reference back to some primary standard
10 (determined by analytical ultracentrifugation).
Pharmica Biosystems supplies the SUPERDEX 75™ column,
which allows for the separation of the various
multimeric forms of self-associating peptides. These
determinations are carried out under non-denaturing
15 (native) conditions and when referenced to the
appropriate standards can be used to identify peptide
and protein oligomerization states.
       As will also be apparent to those skilled in the
art, the test for heterodimerization may be carried
20 out using either of the above two methods or through
the use of CD combined with one or the other of these
methods. This latter technique, in brief, involves
adding known amounts of peptide to a solution
containing a known amount of either the same peptide
25 (for homodimerization) or a different peptide (for
heterodimerization) and following the CD signal as a
function of this addition. An increase in the
magnitude of the signal as peptide is added indicates
that the added material is participating in multimer
30 formation. Homo vs heterodimerization is determined
by carrying out this same experiment using FPLC or
ultracentrifugation, which would determine if the
resulting system is either single (hetero) or multi
(homo) component. A second, and particularly
35 preferred, approach to this same end is to conduct a

CD melt on this same sample. If heterodimerization
has occurred, then a single transition corresponding
to the T_m of the heterodimer will be observed (this T_m
value will probably be different from the value for

5 either of the mixture components). If only
homodimerization takes place then two transitions (two
T_m's) will be observed.

A process for inhibiting HIV-induced cell fusion,
as also disclosed herein, comprising contacting to an

10 HIV-infected cell an effective fusion-inhibiting
amount of a peptide as given above. The process may
be carried out in vitro in an aqueous solution, or may
be carried out in vivo in a cellular culture assay for
HIV infection (e.g., the CEM-SS cell monolayer plaque

15 assay described in L. Kucera et al., Aids Research and
Human Retroviruses 6, 491 (1990) or in an animal
subject afflicted with the HIV virus- The process may
be carried out with peptides of the invention in the
form of multimers (particularly dimers) thereof as

20 discussed above. The process may be carried out in a
human or animal subject to prevent HIV-induced cell
fusion, in which case the compounds may be combined
with a suitable pharmaceutically acceptable carrier
(such as sterile, pyrogen-free physiological saline

25 solution, or sterile, pyrogen free phosphate-buffered
saline solution), and administered to the subject by a
suitable route (i.e., by intramuscular injection,
subcutaneous injection, or intravenous injection).
The therapeutic dosage is about 1 to 10,000 µg/Kg of

30 patient weight per day, more particularly from about
10 to 1,000 µg/Kg of patient weight per day, and most
particularly about 100 µg/Kg of patient weight per
day. Thus, the present invention provides a method of
combatting HIV (and particularly HIV-induced cell

35 fusion) in a human or animal subject by administering

an active compound as given herein in an effective in
an effective HIV (or more particularly HIV-induced
cell fusion)-combatting amount. The present invention
also provides the use of an active compound as given
5 herein for the preparation of a medicament for
combatting HIV (or more particularly HIV-induced cell
fusion) in a human or animal subject in need of such
treatment.

     A still further aspect of the present invention
10 is a process, useful in rational drug design, for
testing compounds for the ability to inhibit the
ability of HIV to infect cells. The process comprises
(a) contacting a test compound to a multimer (e.g.,
dimer, tetramer) of a peptide as given above, and then
15 (b) detecting whether the test compound disrupts said
multimer, the ability of said test compound to disrupt
said multimer indicating the test compound is capable
of inhibiting HIV infection of cells. This process is
noteworthy for its ability to identify compounds
20 directed to a site which has not heretofore been
explored in rational drug design. The process may be
conveniently carried out in vitro in an aqueous
solution containing the multimer by adding the test
compound to the aqueous solution, and then determining
25 whether or not the multimer structure has been
disrupted. Disruption of multimer structure may be
determined in the same manner as set forth above.

     Note that amino acid sequences disclosed herein
are presented in the amino to carboxy direction, from
30 left to right. The amino and carboxy groups are not
presented in the sequence. Amino acids are
represented herein by one letter code or three letter
code as follows:

          Ala; A=Alanine   Leu; L=Leucine
35   Arg; R=Arginine   Lys; K=Lysine

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          Asn; N=Asparagine          Met; M=Methionine
          Asp; D=Aspartic acid       Phe; F=Phenylalanine
          Cys; C=Cysteine            Pro; P=Proline
          Gln; Q=Glutamine           Ser; S=Serine
     5    Glu; E=Glutamic Acid       Thr; T=Threonine
          Gly; G-=Glycine            Trp; W=Tryptophan
          His; H=Histidine           Tyr; Y=Tyrosine
          Ile; I=Isoleucine          Val; V=Valine
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10   The foregoing abbreviations are in accordance with
        established usage. See, e.g., U.S. Patent No.
        4,871,670 to Hudson et al. at Col. 3 lines 20-43
        (applicants specifically intend that the disclosure of
        this and all other patent references cited herein be
15   incorporated herein by reference).
            The present invention is explained in greater
        detail in the following Examples. These Examples are
        for illustrative purposes only, and are not to be
        taken as limiting of the invention.
20

                6. EXAMPLE: Peptide Synthesis
            Peptides DP-107 (SEQ ID NO: 1) , DP121 (SEQ ID
        NO: 2), DP-125 (SEQ ID NO: 3), DP-116 (SEQ ID NO: 4),
        and DP-31 (SEQ ID NO: 5) were synthesized using FAST
25   MOC™ chemistry on an Applied Biosystems Model 431A
        peptide synthesizer. Amidated peptides were prepared
        using Rink resin (Advanced Chemtech) while peptides
        containing free carboxy termini were synthesized on
        Wang (p-alkoxy-benzyl-alcohol) resins (Bachem). First
30   residues were double coupled to the appropriate resin
        and subsequent residues were single coupled. Each
        coupling step was followed by acetic anhydride
        capping. Peptides were cleaved from the resin by
        treatment with TFA (10ml), H_2O (0. 5ml), thioanisole
35   (0.5ml), ethanedithiol (0.25ml), and crystalline

phenol (0.75g). Purification was carried out by
reverse phase HPLC. Approximately 50 mg samples of
crude peptide were chromatographed on a Waters DELTA
PAK~ C18 column (19mm x 30cm, 15m spherical) using a
5 linear gradient: H_2O/acetonitrile 0. 1% TFA.
Lyophilized peptides were stored desiccated and
peptide solutions were made in water at about 5 mg/ml.
Peptides stored in solution were stable for an
extended period of time at 4°C and could be repeatedly
10 frozen and thawed with little apparent effect on
biological activity.

       The amino acid sequences of the peptides
synthesized are shown in Fig. 1. DP-107 is a 38 amino
acid peptide corresponding to residues 558 to 595 of
15 the HIV-1 TM protein. Gallaher et al. (AIDS Res. and
Human Retro. 5, 431 (1989)) and Delwart et al. (AIDS
Res. and Human Reix-o. 6, 703 (1990)) observed that
the primary sequence of this region was strongly
predictive of a helical secondary structure and also
20 contained a "leucine zipper" repeat. The amino
terminus of the peptide was acetylated and the carboxy
terminus amidated to reduce unnatural charge effects
at those positions. DP-107 and each of the other
peptides used in this study were purified by reverse
25 phase HPLC and in each case the purified peptides gave
a single symmetrical peak by analytical HPLC. The
identity of each peptide was confirmed by electro-
spray mass spectrometry, which yielded the following
results: DP-107: 4526.71 (calculated 4526.31); DP-121:
30 4510.75 (calculated 4510.27) ; DP-116: 2057.32
(calculated 2056.44); DP-125: 4743.46 (calculated
4743.55); DP-31: 2479.35 (calculated 2480.93).

       7. EXAMPLE: Evidence for Secondary Structure in
                     Solution
35

Circular dichroism spectra were measured in 10 mM
sodium phosphate, 150 mM sodium chloride, pH 7.0
buffer at approximately 10 mM concentrations using a 1
cm pathlength cell on a Jobin/Yvon Autodichrograph
5   Mark V CD spectrometer. Peptide concentrations were
determined from A_{280} using Edlehoch's method
(Biochemistry 6, 1948 (1967)).

A summary of the ultraviolet CD analyses of
DP-107 is shown in Fig. 2. The results suggest a
10 considerable amount of secondary structure for the
peptide under physiologic conditions. The double
minima at 222 and 208nm are characteristic of alpha
helices and mean molar ellipticity values ($[Q]_{222}$) of
-32,000 at 0°C (Fig. 2A) and -27,000 at 37°C (Fig. 2B)
15 indicate that the peptide is approximately 100% and
85% folded at these temperatures (Y. Chen et al.,
Biochemistry 13, 3350 (1974); N. Greenfield & G.
Fasman, Biochemistry 8, 4108 (1969)). The stability
of the observed structure is illustrated by the
20 thermal melt data shown in Fig. 2C. For example, at a
10 mM concentration of DP-107, the midpoint of the
melting curve (T_m) was approximately 72°C. Also
apparent in Figure 2C is that the T_m for DP-107 varies
as a function of peptide concentration. This
25 concentration dependence is characteristic of leucine
zipper-type structures and is indicative of
stabilization of secondary structural elements by
self-association (E. O'Shea et al., Science 243, 538
(1989)). Oligomerization of DP-107 in solution to
30 form dimers and tetramers is also suggested by
sedimentation equilibrium studies. Taken together,
the results shown in Fig.. 2 tend to support the
predictions (W. Gallaher et al., supra; E. Delwart et
al., supra) that the region of gp41 corresponding to
35 DP-107 contains a leucine zipper-like (coiled coil)

motif which may play a role in envelope
oligomerization. This type of structure can be
described as a homodimer formed by the specific (and
often parallel) association of two alpha helices.

5   This interaction is characterized by the alignment of
the hydrophobic faces of the helices. The unusually
stable secondary structure exhibited by peptides
involved in these types of systems is due to these
higher- order interactions. Preliminary analysis of

10   the solution structure of DP-107 by multi-dimensional
nuclear magnetic resonance (NMR) spectroscopy
indicates a large number of sequential NH-NH$_i$
crosspeaks in the NOESY spectra which is consistent
with the CD evidence that under physiologic

15   conditions, the peptide exhibits significant a-helical
secondary structure.

     The CD spectra of two other synthetic peptides
are also shown in Fig. 2. One of these (DP-121) is
identical to DP-107 but with the isoleucine at

20   position 578 replaced with a proline residue. The
other peptide, DP-116, is a 17mer and overlaps the
carboxy terminus of DP-107. This peptide was
synthesized to contain the same amino acids and
blocking groups as CS3, a peptide described by Qureshi

25   et al. (AIDS 4, 553 (1990)) and reported to exhibit
anti-viral activity when coupled to albumin. The CD
spectra observed for these two peptides indicate that
both exist in random coil conformations at 37°C in
direct contrast to the results obtained for DP-107.

30   This outcome was expected for the proline substituted
analogue, DP-121, in that the proline residue would
tend to both break helix formation as well as disrupt
hydrophobic interactions thought to stabilize coiled
coil structures.

35

8.   EXAMPLE: Reverse Transcriptase (RT) Assay

The micro RT assay was adapted from Goff et al.
(J. Virol. 38, 239 (1981)) and Willey et al. (J.
Virol. 62, 139 (1988)). Supernatants from virus/cell
5   cultures are made 1% in Triton-X100. A 10ml sample of
supernatant was added to 50ml of RT cocktail in a 96
well U bottom microtiter plate and the samples
incubated at 370C for 90 min. The cocktail contained
75mM KCl, 2mM dithiothreitol, 5mm Mgcl21 5mg/ml poly A
10 (Pharmacia cat. No. 27-4110-01), 0.25 units/ml oligo
dT (Pharmacia cat. No. 27-7858-01), 0.05% NP40, 50mM
Tris-HCl, pH 7.8, 0.5 mM non-radioactive dTTP, and 10
mCi/ml ^{32}p-dTTP (Amersham cat. No. PB.10167). After
the incubation period, 40 ml of reaction mixture was
15 applied to a Schleicher and Schuell NA45 membrane (or
DE81 paper) saturated in 2xSSC buffer (0.3M NaCl and
0.003M sodium citrate) held in a Schleicher and
Schuell Minifold over one sheet of GB003 filter paper.
Each well of the minifold was washed four times with
20 200 ml 2xSSC. The membrane was removed from the
minifold and washed 2 more times in a pyrex dish with
an excess of 2xSSC. Finally the membrane was drained
on absorbent paper, placed on Whatman #3 paper,
covered with saran wrap, and exposed to film
25 overnight.

9.   EXAMPLE: HIV-1 Virus Propagation

The HIV-1$_{LAI}$ virus was obtained from R. Gallo (see
M. Popovic et al., Science 224, 497 (1984)) and
30 propagated in CEM cells cultured in RPMI 1640
containing 10-% FCS. Supernatant from the infected
CEM cells was passed through a 0.2 mm filter and the
infectious titer estimated in a microinfectivity assay
using the AA5 cell line to support virus replication.
35 For this purpose 25 ml of serially diluted virus was

added to 75 ml AA5 cells at 2×10^5/ml in a 96 w 11
microtiter plate. Each virus dilution was tested in
triplicate. Cells were cultured for eight days by
addition of fresh medium every other day. On day 8

5   post infection supernatant samples were tested for
virus replication as evidenced by RT activity released
to the supernatant in accordance with the procedure
described above. The $TCID_{50}$ was calculated according
to the Reed and Muench formula in accordance with ·

10   known techniques. See L. Reed et al., Amer. J.
Hygiene 27, 493 (1938) . The titer of the $HIV-1_{LAI}$
stock used for these studies, as measured on the AA5
cell line, was approximately $1 \times 10^7 TCID_{50}$/ml. The two
primary isolates were obtained from PBMCs of two

15 · infected donors, one from Brazil ($HIV-1_{Br3}$) and the
other Trinidad ($HIV-1_{QZ2775}$) by co-cultivation with PHA-
blasted normal donor PBMCs in RPMI 1640 containing
IL2. The infectious titers of the primary virus
stocks were estimated by titration onto normal human ·

20   PHA blasted PBMCs in a 96 well microtiter plate, again
using RT activity released to the supernatant as
evidence for successful infection. The infectious
titer of both of these isolates was estimated to be
approximately $1 \times 10^3 TCID_{100}$/ml.

25

   10.   EXAMPLE: Peptide Inhibition of Infected Cell
                  Induced Syncytia Formation

        The initial screen for antiviral activity of the
peptides shown in Fig. 1 was for blockade of syncytium

30   formation induced by overnight co-cultivation of
uninfected Molt4 cells with chronically infected (HIV-
1_{IIIB}) CEM cells.

        Approximately 7×10^4 Molt cells were incubated
with 1×10^4 CEM cells chronically infected with the HIV-

35   1_{LAI} virus in 96 well plates (one-half area cluster

plates;Costar. Cambridge, MA) in a final volume of
100ml culture media in accordance with known
techniques (T. Matthews et al., Proc. Natl. Acad.
Sci. U. S. A. 84, 5424 (1987)). Peptide inhibitors

5   were added in a volume of 10ml and the cell mixtures
were incubated for 24 hr at 37°C. At that time point,
multinucleated giant cells were estimated by
microscopic examination at a 40x magnification which
allowed visualization of the entire well in a single

10   field.

The results of three such experiments are
shown in Table 1. In the first of these, serial
peptide concentrations between 50 ㎍/ml and 1.5 mg/ml
were tested for blockade of the cell fusion process.

15   It is shown that DP-107 afforded complete protection
down to a concentration of 6 mg/ml. The overlapping
17mer peptide, DP-116, which is analogous to the
previously described CS3 by Qureshi et al. (AIDS 4,
553 (1990)) exhibited no evidence of anti-fusogenic

20   activity even at 50 mg/ml. This observation is in
agreement with that study which only found anti-viral
activity for CS3 after conjugation to albumin. A
second peptide DP-31 representing an overlapping
immunodominant site (M. Oldstone et al., J. Virol. 65,

25   1727 (1991); J. Wang et al., Proc. Natl. Acad. Sci.
USA 83, 1659 (1986)) also failed to show inhibitory
activity.

30

35

TABLE 1. Test for Peptide Blockade of HIV-1 Induced Cell-Cell Fusion

		Syncytia Number Peptide Concentration (micrograms/ml)						
	Peptide	50	25	12	6	3	1.5	0
EXP. 1	DP-31	85	80	78	87	90	75	89
	DP-116	89	82	93	92	89	82	89
	DP-107	0	0	0	0	46	80	89
		40	20	10	5	2.5	1.25	0
EXP. 2	DP-107	0	0	0	36	83	98	93
	DP-125	0	0	0	0	3	35	93
EXP. 3	DP-121	69	71	65	60	68	ND	76
	DP-125	0	0	0	0	0	0	76

The inhibitory activity of DP-107 did not appear related to cytotoxic or cytostatic effects since in other studies CEM cells grown in the presence of DP-107 at 50 mg/ml (the highest concentration tested) for three days with fresh peptide added each day displayed the same viability and growth rate as control cultures. We also found that DP-107 blocked fusion mediated by the other prototypic isolates: HIV-$1_{MN, RF,}$ and $_{SF2}$.

The concentration dependence of the CD spectra described suggests that the structure of the DP-107 is stabilized by peptide self association. In similar studies, O'Shea et al. (Science 243, 538 (1989)) reported that disulfide bridging of a peptide of the leucine zipper domain in the GCN4 protein (a transcriptional regulatory factor) to form covalently bonded homodimers stabilized the coiled coil structure. Following similar reasoning we sought to determine if the limiting effective concentration for cell fusion blockade by DP-107 might in part be related to the concentration dependence of peptide

self-association. In order to test this possibility
we synthesized a DP-107 analogue with a cystein
containing "tail" which after purification could be
air oxidized to yield a homodimer. The resulting
5 peptide, DP-125, exhibited about twice the apparent
molecular weight of DP-107 in SDS-PAGE under non-
reducing conditions indeed suggesting that a
covalently bonded homodimer was generated. In the
syncytial blocking assays (Table 1 experiments 2 and
10 3) this analogue was, in fact, more efficacious than
DP-107, requiring one-half to one-fourth the
concentration for inhibition. The increased activity
exhibited by DP-125 has reproduced in all assays
performed to date and suggests that a dimer or higher
15 order multimer might actually represent the
biologically active form of the peptide. Also, CD
measurements of DP-125 were found to yield similar
ellipticity values as the parental DP-107. Taken
together these observations indicate that the
20 individual peptide components of the putative
multimers are arranged in a parallel rather than anti-
parallel orientation.
      To gain further insights as to whether the
solution structure observed for DP-107 in the CD and
25 NMR studies is required for biological activity, the
proline-containing analog (DP-121) which failed to
exhibit helix related signals at 37°C in the CD
experiments was tested for activity in the cell fusion
assay. The results showed no sign of inhibitory
30 activity as indicated in Table 1. Although this does
not prove that structure is necessary for biological
activity, it is consistent with that possibility. In
a similar fashion, each of the DP-107 peptide
analogues tested to date that failed to block cell
35 fusion have also failed to show evidence of stable

solution structure in CD studies. Also, a peptide
which formed a coiled coil structure (GCN4-p1,
provided by R. Rutkowski) exhibited no biological
activity.

11. EXAMPLE: Peptide Inhibition of Infection by
                Cell-Free Virus

The peptides were next tested for blockade of
infection by cell-free virus. The results shown in
Fig. 3 are representative of several experiments in
which the DP-107, 125, and 116 (CS3) peptides were
compared for potency in the blockade of HIV_{LAI},
infection of AA5 cells. Each level of peptide was
incubated in triplicate with about 500 $TCID_{50}$ of virus
and cells. After 7 days of culture, cell-free
supernatant was tested for the presence of RT activity
as a measure of successful infection. The results are
shown in Fig. 3 and demonstrate that both the DP-107
and DP-125 reagents inhibited the de-novo infection
process at about the same effective doses as noted in
the fusion assays described above. Moreover the dose
required for inhibitory effect was lower for the
disulfide bridged DP-125 analogue and no hint of anti-
viral effect was noted for the DP-116 peptide.

12. EXAMPLE: Peptide Inhibition of PBMC Infection
                by Primary Isolates

It is now apparent that substantial differences
in sensitivity to anti-viral agents can exist between
laboratory adapted prototypic isolates and primary
field isolates passaged only through PBMCs. This
problem was first highlighted by Ho and colleagues (E.
Dear et al., Proc. Natl. Acad. Sci. 87, 6574 (1990))
in studies with soluble CD4. To test if the gp41
peptides might exhibit a similar discordance in
reactivity, the DP-107, DP-125 (cysteine analogue),

and sT4 (same reagent as used in the preceding
exp riment) were compared for inhibition of PBMC
infection by two primary HIV-1 isolates and HIV_{LAI}.
The results of these studies (Fig.5) show that the
5 peptides inhibit both the primary and prototypic
isolates tested. Only a single dose of sT4 (10 mg/ml)
was included for comparative purposes and it is
apparent that this reagent is substantially more
active on the cell-line adapted $HIV-1_{LAI}$ virus in
10 comparison to the two primary isolates.

        13.   EXAMPLE: Synthesis of DP-107 Analogs
        Analogs of DP-107 were synthesized in accordance
with known techniques as given in Example 1 above.
15 Such analogs are set forth in Table 2 below.

    TABLE 2.   DP-107 Analogs.

DP-118	QQLLDVVKRQQEMLRLTVWGTKNLQARVTAIEKYLKDQ	(SEQ ID NO:5
DP-122	GIKQLQARILAVERYLKDQQ	(SEQ ID NO:6
DP-123	IEAQQHLLQLTVWGIKQLQARILAVERYLKDQ	(SEQ ID NO:7
DP-127	NNLLRAIEAQQHLLQLTVWGIKQLQARILAV	(SEQ ID NO:8
DP-129	NNLLRAIEAQQHLLQLTVWGIKQLQARILAVERYLKDQGGC	(SEQ ID NO:9
DP-130	CGGNNLLRAIEAQQHLLQLTVWGIKQLQARILAVERYLKDQGGC	(SEQ ID NO:10

- 23 -

14. EXAMPLE: Activity of DP-107 Analogs

The activity of various DP-107 analogs as described herein was tested by the syncytia assay described in Example 5 above. These data are given in Table 3 below.

TABLE 3

Peptide	[CONC]	No. Syncytia/well at peptide concentration, ug/ml on IIIB						
		40	20	10	5	2.5	1.25	0
DP116	8.0 mg/ml	77						
DP107	4.5 mg/ml	0	0	0	4	68	88	
DP118	3.9 mg/ml	0	0	77	80	78	74	
DP121	3.9 mg/ml	79						
DP122	3.4 mg/ml	73						
DP123	4.0 mg/ml	4	55	86	76	69	80	
DP125	3.1 mg/ml	0	0	0	0	0	0	
DP127	3.3 mg/ml	0	0	0	20	53	87	
DP129	3.4 mg/ml	0	0	0	0	0	0	
DP130	4.5 mg/ml	0	0	0	0	0	47	
Ctrl cells only								85

15. EXAMPLE: Biological Activity of DP-107 and Analogs Thereof

The biological activity of DP-107 and various analogs thereof in the fusion assay described in Example 5 above and the neutralization assay described in Example 6 above is summarized in Table 4 below. The quantity of compound required to produce a ninety percent reduction in number of syncytia is shown in the column marked "Fusion"; the quantity of compound required to produce a ninety percent reduction in infectivity is given in the column marked "Neutraliz". Compounds active in the fusion assay at a range between 20 and 40 4 µg/ml are considered moderately active; compounds active at a range between 10 and 20 µg/ml are considered potent; and compounds active in

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an amount of less than 10 μg/ml are considered very
potent.

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TABLE 4

BIOLOGY OF DP-107 AND ANALOGS

		Fusion [ug/ml]¹	Neutraliz [ug/ml]²
DP-107 (SEQ ID No: 1)	NNLLRAIEAQQGLLQLTVWGIKQLQARILAVERYLKDQ	5	10
DP-116 (SEQ ID No: 1)	LQARILAVERYLKDQQL	>40	>30
DP-121 (SEQ ID No: 4)	NNLLRAIEAQQGLLQLTVWGIKQLQARILAVERYLKDQ	>40	>30
DP-122 (SEQ ID No: 2)	GIKQLQARILAVERYLKDQQ	>40	>?0
DP-123 (SEQ ID No: 6)	IEAQQGLLQLTVWGIKQLQARILAVERYLKDQ	40	>?0
DP-125 (SEQ ID No: 7)	CGGNNLLRAIEAQQGLLQLTVWGIKQLQARILAVERYLKDQ	<1	2
DP-127 (SEQ ID No: 8)	NNLLRAIEAQQGLLQLTVWGIKQLQARILAV	7	23
DP-129 (SEQ ID No: 9)	NNLLRAIEAQQGLLQLTVWGIKQLQARILAVERYLKDQGGC	<1	<1
DP-130 (SEQ ID No: 10)	CGGNNLLRAIEAQQGLLQLTVWGIKQLQARILAVERYLKDQGGC	2	<1
DP-136 (SEQ ID No: 11)	CGGNNLLRAIEAQQGLLQLTVWGIKQLQARILAV	>40	>30
DP-137 (SEQ ID No: 12)	LSGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARILAV	12	12
DP-138 (SEQ ID No: 13)	CGGLSGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARILAV	>40	>30
DP-139 (SEQ ID No: 14)	NNLLRAIEAQQHLLQLTVWGIKQLQARILAVERYLKDQ	>40	>30
DP-140 (SEQ ID No: 15)	NNLLRAIEAQQHLLQLTVWGIKQLQARILAVERYLKDQ	17	14

[1] 90% reduction in number of syncytia (control=90)
[2] 90% reduction in infectivity
Bold letters=non-naturally occurring amino acid residues

The foregoing examples are illustrative of the present inv ntion, and are not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be

5   included therein.

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What is Claimed Is:

    1.   An isolated peptide comprising a DP-107 amino acid sequence listed in SEQ ID:1.

5

    2.   An isolated peptide ranging from about 14 to about 60 amino acids in length, capable of forming a heterodimer with the peptide of Claim 1.

10   3.   The peptide of Claim 1 or 2 wherein the amino terminus of the peptide is acetylated.

    4.   The peptide of Claim 1 or 2 wherein the carboxy terminus of the peptide is amidated.

15

    5.   An isolated multimer of the peptide of Claim 1 or 2.

    6.   The multimer of Claim 5 wherein the multimer

20 is a tetramer.

    7.   The multimer of Claim 5 wherein the multimer is a dimer consisting of two peptide monomers.

25   8.   The dimer of Claim 6 wherein the monomers of the dimer are covalently bound to one another.

    9.   A method for inhibiting HIV-induced cell fusion comprising contacting an HIV-infected cell with

30 an effective amount of a peptide comprising a DP-107 amino acid sequence listed in SEQ ID:1 so that the cell fusion is inhibited.

    10.   The method of Claim 9 wherein the HIV is

35 HIV-1.

- 28 -

11. A method for inhibiting HIV-induced cell
fusion comprising contacting an HIV-infected cell with
an effective amount of a peptide comprising the
peptide of Claim 2 so that the cell fusion is
5 inhibited.

12. The method of Claim 11 wherein the HIV is
HIV-1.

10   13. The method of Claim 9 wherein the peptide is
present as a multimer.

14. The method of Claim 11 wherein the peptide
is present as a multimer.
15

15. The method of Claim 13 or 14 wherein the
multimer is a dimer having two peptide monomers.

16. The method of Claim 12 wherein the monomers
20 are covalently bound to one another.

17. A method for testing compounds capable of
inhibiting the ability of HIV to infect cells,
comprising:
25   (a)   contacting a test compound to a multimer of
a peptide comprising a DP-107 amino acid sequence
listed in SEQ ID:1; and
(b)   detecting whether the test compound disrupts
the multimer, the ability of the test compound to
30 disrupt the multimer indicating the test compound is
capable of inhibiting HIV infection of cells.

18. The method of Claim 17 wherein the HIV is
HIV-1.
35

19. The method of Claim 17 wherein the multimer
is a dimer or a tetramer.

20. The method of Claim 17 wherein the
5 contacting step is carried out in an aqueous solution.

21. A method for testing compounds capable of
inhibiting the ability of HIV to infect cells,
comprising:
10   (a) contacting a test compound to a multimer of
the peptide of Claim 2; and
          (b) detecting whether the test compound disrupts
the multimer, the ability of the test compound to
disrupt the multimer indicating the test compound is
15 capable of inhibiting HIV infection of cells.

22. The method of Claim 21 wherein the HIV is
HIV-1.

20   23. The method of Claim 21 wherein the multimer
is a dimer or a tetramer.

24. The method of Claim 21 wherein the
contacting step is carried out in an aqueous solution.
25

25. A method for inhibiting enveloped viral
infection comprising contacting an uninfected cell
with an effective amount of a peptide capable of
contributing to the formation of a coiled coil peptide
30 structure so that an enveloped virus is inhibited from
infecting the uninfected cell.

26. The method of Claim 25 wherein the enveloped
virus is a retrovirus.
35

27. The method of Claim 26 wherein the
retrovirus is HIV-2, HTLV-I, or HTLV-II.

28. The method of Claim 25 wherein the enveloped
virus is an influenza virus.

29. The method of Claim 25 wherein the enveloped
virus is a respiratory syncytial virus.

29. A method for testing compounds capable of
inhibiting the ability of an enveloped virus to infect
cells, comprising:
     (a) contacting a test compound to a multimer of
a peptide capable of contributing to the formation of
a coiled coil peptide structure; and
     (b) detecting whether the test compound disrupts
the multimer, the ability of the test compound to
disrupt the multimer indicating the test compound is
capable of inhibiting enveloped viral infection of
cells.

30. The method of Claim 29 wherein the enveloped
virus is a retrovirus.

31. The method of Claim 30 wherein the
retrovirus is HIV-2, HTLV-I, or HTLV-II.

32. The method of Claim 29 wherein the enveloped
virus is an influenza virus.

33. The method of Claim 29 wherein the multimer
is a dimer or a tetramer.

34. The method of Claim 29 wherein the
contacting step is carried out in an aqueous solution.

550'

p.107   NNLLRAIEAQQHLLQLTVWGIKQLQARILAVERYLKDQ

p.121   NNLLRAIEAQQHLLQLTVWGI[P]KQLQARILAVERYLKDQ

p.125   CGGNNLLRAIEAQQHLLQLTVWGIKQLQARILAVERYLKDQ

609'

p.31   ERYLKDQQLLGIWGCSCKLICG

p.116   LQARILAVERYLKDQQQ

Fig 1
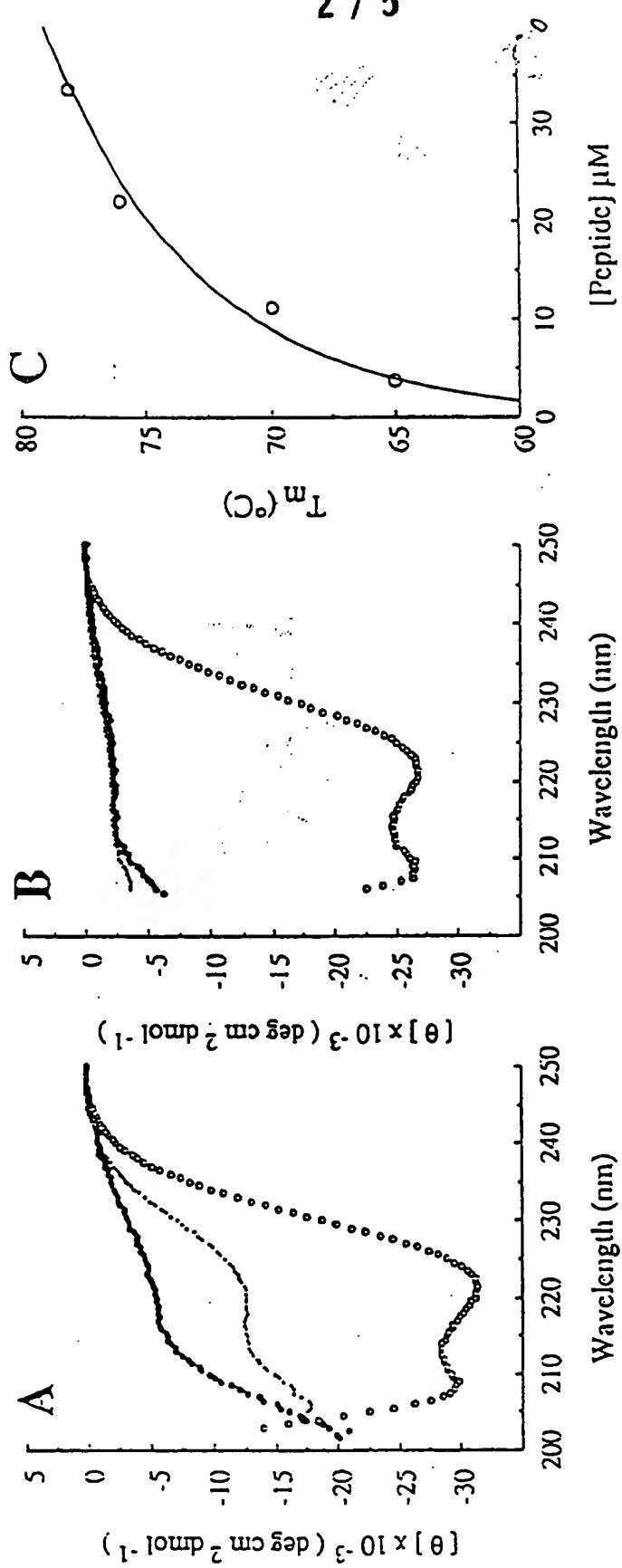
Fig 2.
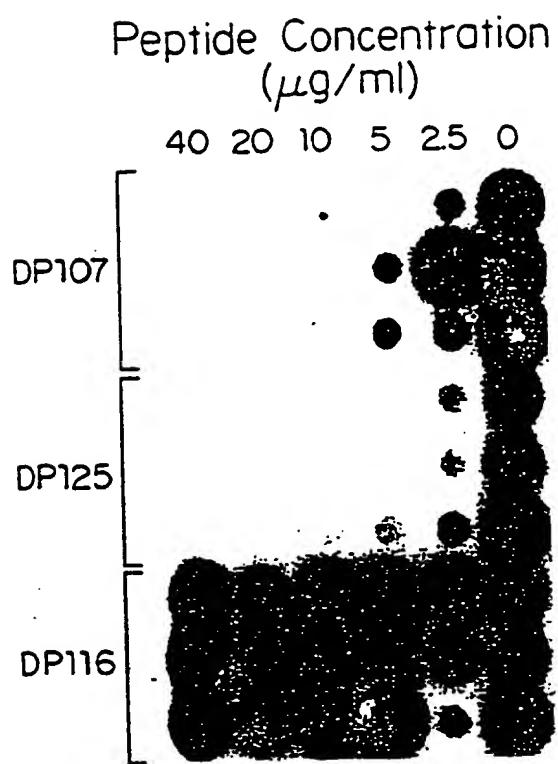
Fig 3.
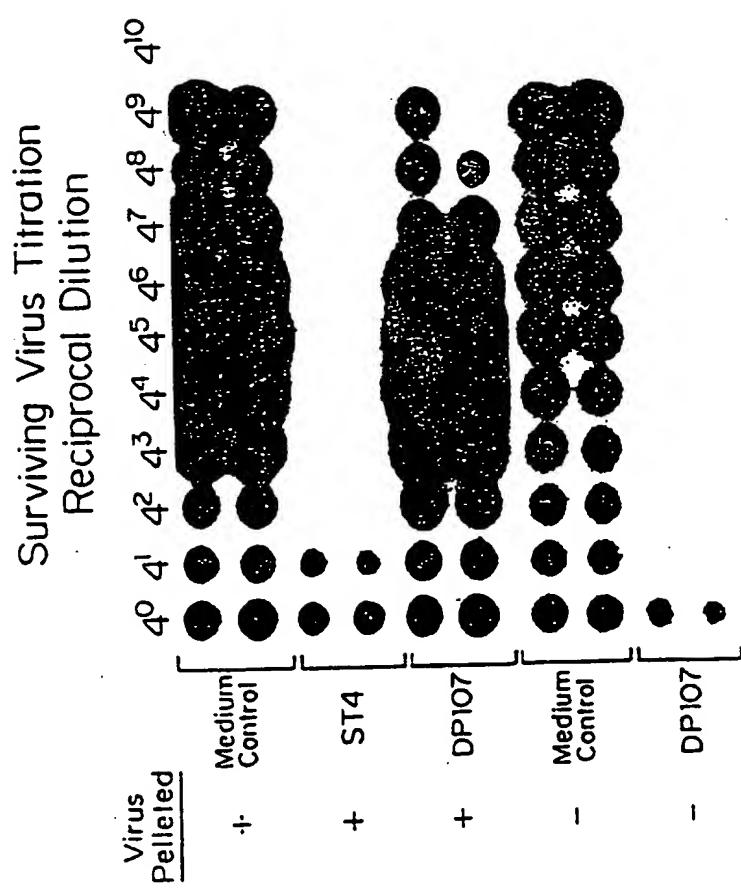
Fig 4
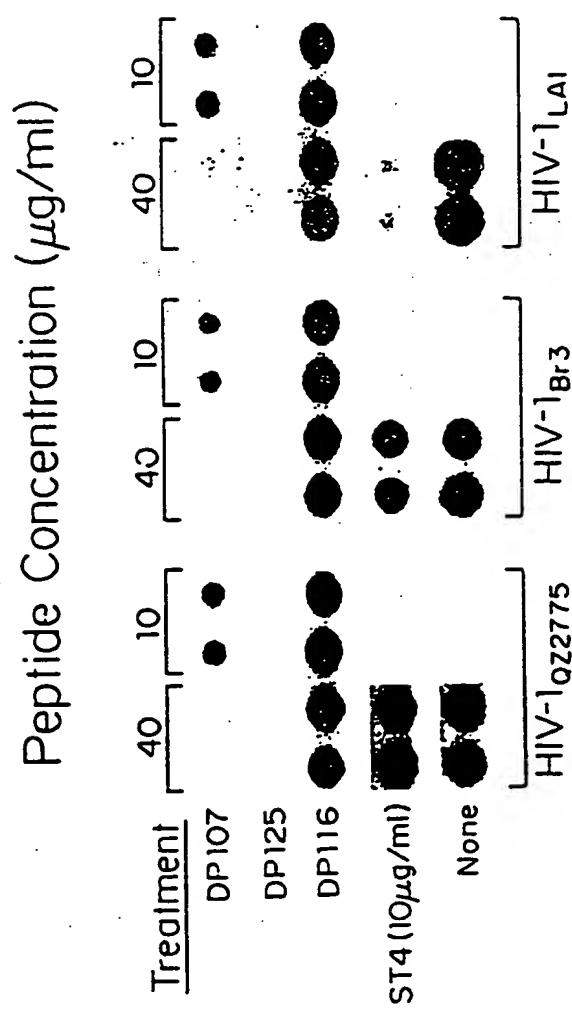
Fig 5

International application No.

PCT/US93/06769

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07K 07/00, A61K 37/02
US CL :514/012; 530/324

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/012; 530/324

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, MEDLINE, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	AIDS Research and Human Retroviruses, Volume 5, Number 4, issued 1989, Gallaher et al. "A General Model for the Transmembrane Proteins of HIV and Other Retroviruses", pages 431-440, see entire document.	1-8
Y	AIDS Research and Human Retroviruses, Volume 6, Number 6, issued 1990, Delwart et al., "Retroviral Envelope Glycoproteins Contain a 'Leucine Zipper'-like Repeat", pages 703-706, see entire document.	1-8

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 September 1993	OCT 05 1993

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	MERRELL C. CASHION
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Form PCT/ISA/210 (second sheet)(July 1992)*

International application No.

PCT/US93/06769

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	The Journal of General Virology, Volume 73, Part 7, issued July 1992, Buckland et al., "A Leucine Zipper Structure Present in th Measles Virus Fusion Protein is not Required for its Tetramerization but is not Essential for Fusion", pages 1703-1707, see the abstract.	1-8